EXHIBIT 2

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Clinical Report

Duplication of (2)(q11.1-q13.2) in a Boy With Mental Retardation and Cleft Lip and Palate: Another Clefting Gene Locus on Proximal 2q?

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A 4-year-old boy with left cleft lip and cleft palate, multiple minor anomalies and developmental delay revealed an abnormal chromosome 2 with enlarged proximal long arm, de novo, in his karyotype. Fluorescence in situ hybridization with a chromosome 2 library and band-specific YACs confined the duplicated segment to 2q11.1-q13.2 and indicated a direct tandem duplication due to unbalanced crossover between chromatids.

KEY WORDS: **cleft lip and palate; multiple anomalies/mental retardation syndrome; partial trisomy 2q(q11-q13); tandem duplication 2q**

INTRODUCTION

While partial trisomy of segments of the distal long arm of chromosome 2 are well known from a number of reports, proximal duplications were rarely reported because their determination requires successive FISH and molecular investigations. The clinical picture of the few reported cases as well as the breakpoints determined by the different authors vary considerably, and hence no common pattern of a proximal duplication 2q syndrome can yet be defined. Cleft lip and palate is not a consistent finding.

We report a further case of a de novo proximal 2q tandem duplication identified with the help of fluorescence in situ hybridization studies using band-specific YAC probes in a patient with unilateral cleft lip and cleft palate, but otherwise only discrete minor anomalies associated with developmental delay.

CLINICAL REPORT

The propositus is a 4-year-old boy. He was the product of the second pregnancy to a healthy 30-year-old mother and her healthy 31-year-old husband. The pedigree is unremarkable. The first child, a boy, was born at term with 3.870 g and 52 cm length and so far developed normally. A younger brother was delivered at 39 weeks of gestation with a weight of 2.100 g (< 10th centile), length 47 cm (10th centile), and OFC 31.5 cm (< 10th centile). The propositus' fetal activity, first noticed at 20 weeks of gestation, was decreased as compared to the previous pregnancy, but otherwise the gestation was unremarkable; three ultrasound routine examinations did not disclose abnormal findings. Delivery occurred 10 days past the expected term; birth weight was 2.900 g (10th centile), length was 47 cm (< 10th centile). A left cleft lip with cleft palate and a natal left upper incisor were noted at birth.

Developmental milestones were delayed with free sitting at 12 months, first free steps at 20 months, and first words at 24 months. According to his physiotherapist, fine motor skills are especially delayed and defective. At age 6 months, the cleft lip, and at age 18 months, the cleft palate were operated on. Correction of the maxillary cleft is forseen for the age of 4 years. He had suffered from repeated otitis and on hearing tests showed moderate conductive hearing loss.

Although attacks suspicious for absences were already observed early after birth, the first longer (2–3 min) absence fit occurred only at age 2 years 3 months. EEGs did not show epileptic activities, and magnetic resonance showed only a mild hypoplasia of the hippocampus area. Following repeated longer absences, he was put on Depakine (Valproate), which resulted in a decrease of both frequency and duration of the fits, which subsequently occurred about every 3 weeks and ceased at age 3 years.

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Fig. 1. Face of the propositus at age 2 years 9 months. Note high frontal hairline, asymmetric nose with short columella and prominent bridge, and repaired cleft lip.

At examination at age 2 years 6 months (Fig. 1), length was 89.6 cm (25th centile), weight was 12.5 kg (3rd centile), and head circumference was 47.5 cm (3rd centile). He had minor dysmorphisms: a high frontal hairline with a cowlick, bluish sclerae, no strabismus, no epicanthic folds, mild myopia, a short, broad, and (due to the cleft lip) asymmetric beaked nose with a prominent bridge and a short columella, hypoplastic philtrum, normal ears (ear length, 5.2 cm; 25th centile), tooth eruption normal for age, normal trunk, male genitalia with a penis of average length, a small scrotum and a left inguinal testis, normal hand length (10.3 cm) and middle finger length (4.5 cm), but tapering fingers with clinodactyly of the fifth ray, extension contractures in the metacarpo-phalangeal joints ofthumbs, proximally implanted fifth toes, and dysplasia of hallucal nails.

At the last examination at age 4 years, height was 1.00 m (10th centile), weight was 14.0 kg (3rd centile), and OFC was 50 cm (< 3rd centile). Physical findings were unchanged. He spoke in two-word sentences and had not yet achieved bladder or sphicter control. He did not dress or undress himself and showed poor motor coordination with a high motor activity. He liked music and water and riding in a car. Except for jealousy toward his younger brother, he was easygoing.

RESULTS

Cytogenetics and Fluorescence In Situ Hybridization (FISH) Investigations

Metaphase chromosome preparations were obtained from PHA-stimulated lymphocyte cultures from the patient and both parents according to standard procedures. GTG-banded chromosome examination at a 400–600 band level showed a 46,XX,2q+ karyotype in the patient. The proximal 2q segment seemed to be increased in length. Karyotypes from both parents were normal. FISH analysis on the patient with a chromosome 2–specific paint probe (Vysis, Downers grove, IL) showed uniform hybridization along the entire length of the normal and abnormal chromosome 2, implying noninvolvement of other chromosomes in the rearrangement (data not shown). Yeast artificial chromosomes (YACs) mapping to the presumably duplicated region on the chromosome were used in order to define further the duplicated segment. YAC clones of interest were

TABLE I. Results of FISH Analysis With YAC Probes in the Propositus

FISH probe	Location	Hybridization*
774-e-9	2q11.1	+++
826-e-11	2q11.1	+++
726-e-10	2q12	+++
759-g-6 + 763-g-3	2q12.2-13	+++
679-d-2 + 924-b-11	2q14	++

*++, one signal of the probe present on each of the two chromosomes 2 (signals not duplicated); +++, one signal present on the normal chromosome 2 and two signals present on the abnormal 2.

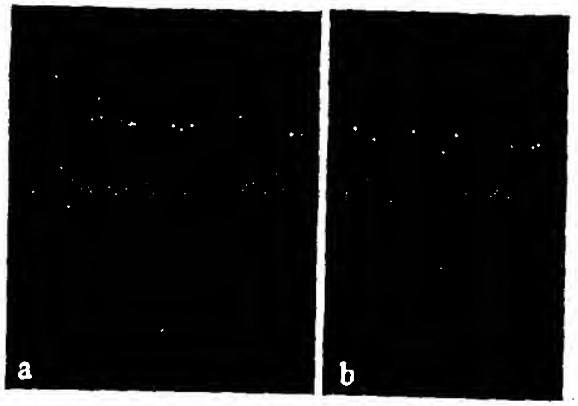

Fig. 2. Partial metaphase from the patient. FISH pattern with YAC (a) y774-e-9 and (b) 759-g-6 probes on the normal chromosome 2 (one green signal) and on the duplicated chromosome 2 (two green signals). The two red signals on each chromosome 2 represent the control probe.

selected from the public databases (http://bioserver.uniba.it/fish/Cytogenetics/) and obtained from the Cytogenetics Unit, Genetics Institute, University of Bari, Italy. The DNA was isolated and used in Alu primed polymerase chain reactions (Alu-PCR) according to standard protocols.

Alu-PCR products were labeled with either biotin-16-dUTP (Boehringer, Mannheim, Germany) or digoxigenin-11-dUTP (Boehringer) by nick translation. Standard protocols were used for hybridization and posthybridization washing. Fluorescein isothiocyanate–conjugated avidin and rhodamine-conjugated antidigoxigenin (Vector Laboratories) were used to detected the probes. Analysis was performed using a Zeiss Axioplan epifluorescence microscope, and images were recorded by a Photometrics CCD KAF1400 camera (Photometrics, Tucson, AZ), controlled with Smart Capture imaging software (Vysis). Vysis Imaging software was also used to convert the DAPI image into G-banded metaphase for identification of the chromosomes.

Cytogenetically, breakpoints in the long arm of chromosome 2 had been assigned at band q11 and q14. Different region-specific YAC probes were used for the identification of the duplication. Table I shows a summary of the FISH analysis, including the localization and the hybridization results of the YAC probes used. Hybridization with y774-e-9 (D2S2209, 116 cM) and y826-e-11 (D2S373, 118 cM) probes, both mapping to chromosome 2 at band q11.1, showed two signals on the duplicated chromosome 2 (Fig. 2a). The YACs 726-d-10 (D2S1893, 125 cM) and y759-g-6 + y763-g-3 (D2S1897, 122 cM), localized to 2q12 and to 2q12.2-q13, respectively, also showed duplicated signals only on the abnormal chromosome 2 (Fig. 2b). However, the YAC probes (679-d-2 + 924-b-11), mapping to chromosome 2 at band q14, showed a normal hybridization pattern for each probe, inferring that these regions were outside of the duplicated segment.

Microsatellite Marker Analysis

Two markers, D2S373 and D2S2209, both mapping to 2q11.1, were analyzed in patient and parents. None of them was informative for heterozygosity from one parent, and no dosage differences could be seen.

DISCUSSION

A comparison of clinical and cytogenetic findings between the propositus of this report and cases of the literature is presented in Table II. Such efforts must be preliminary since most of the cases from the literature were investigated only by banded chromosome examinations, and hence the determination of the duplicated segment is not confident, especially in cases with de novo duplications. This concerns patients 2, 3, and 4. The two affected siblings (cases 5 and 6 from Glass et al. [1998]) and case 7 reported by Lanman et al. [1986] followed familial insertional translocations. However, the segment duplicated in the latter case is more distal and overlaps only for a short part with the segment involved in our propositus. The patients with probably most similar duplicated segments are cases 2 [Wang and Hunter, 1979] and 3 [Mu et al., 1984]. In addition, data from a case with triplication (partial tetrasomy) of probably a similar proximal 2q segment were also included in Table II (case 9, Wang et al. [1999]).

As the evaluation of minor anomalies is difficult and subjective, only features reported in more than one case were included in Table I while major anomalies were listed irrespective of the occurrence. Table I shows that no consistent clinical phenotype is evident from the cases reported thus far of proximal 2q duplication, not even in those cases in whom breakpoints presumably were very similar (cases 2 and 3). This refers both to minor and major anomalies, although it is well known that dysmorphic patterns are much more consistent than major malformations in patients with the same chromosome aberration [Schinzel, 2001]. The only major malformation in our propositus, cleft lip and cleft palate, does not seem to be a consistent feature of any proximal 2q duplication: cleft lip was not reported so far, and cleft palate alone was reported in one patient (case 7 in Table I [Lanman et al., 1986]) with familial duplication involving a more distal segment with a possible slight overlap at 2q13, and in a case with partial tetrasomy—not trisomy—of 2q11-q21.

Nevertheless, occurrence of cleft lip and cleft palate points toward the existence of a gene involved in patterning of the midface including the fusion of the medial nasal process and the maxillary processes and of

TABLE II. Comparison of Clinical Findings in Patients With Duplications/Triplication of Segments Including 2q11-q14*

Case	1	2	3	4	5	6	7	8	9
Duplicated 2q segment	p13-q12	p11-q14	q11.2-q14.2	q11.2-q21	q11.2-q21.1	q11.2-q21.1	q13-q21	q11.1-q13.2	q11.2-q21
Age at last examination (years/months)	0/1	17	3/6	7/4	37	66	0/0	4	0/1
Low birthweight (≤ 10%)		+	−	−				+	
Craniosynostosis		+	+						
Brachycephaly/flat occiput		+	+	+	+				
Microcephaly			+		+				
Low-set ears				+	+	+	+	+	
Prominent ears				+	+				
Preauricular pits							±(r)		
Strabismus		+		+					
Glaucoma			+						
Depressed nasal bridge			+	+					
Wide nasal bridge				+			+		
Cleft upper lip								+	
Cleft palate							+ —	+	+
Small mandible					+	+	+		
Irregular teeth		+						+	
Hypothyroidism						+			
Diaphragmatic hernia	+								
Abnormal position of kidneys							+		
Ureter obstruction						+			
Cryptorchidism								+	
Short nails		+		+				+	
Hyperconvex nails		+						+	
Clinodactyly V			+	+				+	
Arachnodactyly/narrow fingers							+		
Transverse palmar creases							+(r)		+
Clubfoot							+		+
Short toes		+						+	
Polydactyly of toes							−(r)		
Short stature			+		+	+			
Obesity				+	+	+			
Hypertonia/hypotonia		+/−	−/+						
Mental retardation		+	−	−	+	+		+	
Seizures								+	
Aggressivity						+			

*Additional findings: hydrops, anhydramnios, webbed neck, absent right thumb, large clitoris, lung hypoplasia, multicystic kidneys, atretic ureters, hypoplastic bladder, hydrocephalus, absence of septum pellucidum and corpus callosum, Dandy-Walker malformation.

the palatal processes. The observation that no other case had lip and palate clefts is not astonishing. It is in line with the general observation that cleft lip and cleft palate, same as other organ malformations, are rarely consistent features in any given chromosome aberration. Hence, the interaction of other genes as well as different environmental conditions (e.g., placental blood supply during a critical period of organogenesis) may decide whether or not such malformations occur in an embryo. However, even mutation through disruption of a single dominant gene mapping to proximal 2q cannot be excluded as the cause of this malformation in our propositus since the critical break, be it the proximal or distal, could be unique in this patient. To summarize, the observations point toward a gene influencing midface patterning whose mutation or deletion could cause facial clefts mapping to proximal 2, either at 2q11.1 or at 2q13.2. This locus adds to some 100 chromosomal segments so far found in aneuploid state in patients with cleft lip and/or cleft palate [Schinzel, 1994].

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